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# Parametric optimisation of *Aspergillus terreus* lipase production and its potential in ester synthesis

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**Abstract**

Parametric optimisation of lipase from *Aspergillus terreus* yielded 7780 U/l in a medium containing corn oil (2% v/v) and casein (0.1% w/v). Maximum production was observed at pH 9.0 and at 37°C in 96 h. Secretion of lipase was increased by $Ca^{2+}$ and $Mg^{2+}$ ions. The enzyme has good potential for synthesis of fatty acid esters of sugars, sugar alcohols, aliphatic alcohols and ascorbic acid which are industrially important. 

*Keywords:* Lipase; Production; *A. terreus*; Optimisation; Thermostable; Ester synthesis

## 1. Introduction

Lipases (triacyl glycerol ester hydrolase, E.C. 3.1.1.3) catalyse the hydrolysis of fats to produce monoglycerides, diglycerides, free fatty acids and glycerol, with the reaction being reversible in micro-aqueous environments [1,2]. Lipases also possess characteristic properties like substrate specificity, stereospecificity, regiospecificity and the ability to catalyse heterogenous reactions at the interface of water soluble and water insoluble systems and in organic solvents [1,3]. These characteristics have accounted for a marked increase in industrial usage of the enzymes over the last two decades.

During the course of an extensive screening programme for industrially important lipase producers among aspergilli and penicilli [4], *Aspergillus terreus* was found to produce a lipase with the unique property of carrying out deacylation at the ortho-position of peracetylated polyphenolic compounds [5]. In addition, these investigations revealed that *A. terreus* lipase is highly thermostable retaining 100% activity at 60°C for 24 h and activity at a broad pH range, i.e. from 3 to 10 [6]. This lipase is also capable of biosurfactant production from sugar alcohol and natural triglycerides [7]. Owing to the potential applications of this novel enzyme, we report here our findings on process optimisation for maximum lipase production by *A. terreus* and its evaluation for synthesis of esters of fatty acids with sugars, sugar alcohols, aliphatic alcohols and ascorbic acid which are all industrially important.

## 2. Materials and methods

### 2.1. Micro-organism

*Aspergillus terreus*, a natural isolate obtained from soil was grown on potato dextrose agar slants at $37 \pm 1$°C for 4 days and stored at $6 \pm 1$°C in a B.O.D incubator (Caltan) until further use. The culture (RK-101) has been deposited in the culture collection centre of the Department of Microbiology, University of Delhi, South Campus, New Delhi, India.

### 2.2. Lipase production

The enzyme was produced by inoculating 50 ml of Czapek Dox modified minimal medium containing $5 \times 10^7$ spores/ml of 4-day old culture of *A. terreus* in 250 ml Erlenmeyer flasks. The medium contained in grams per litre distilled water: $NaNO_3$, 6; KCl, 0.52;

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$MgSO_4 7H_2O$, 0.52; $KH_2PO_4$, 1.52; $Cu(NO_3)_2 3H_2O$, 0.001; $FeSO_4 7H_2O$, 0.001; $ZnSO_4 7H_2O$, 0.001; glucose, 2.0 and olive oil, 1.5% v/v. Initial pH was adjusted to 6.2. The medium was autoclaved at 121°C (15 psi) for 15 min. The flasks were incubated at $37 \pm 1$°C for 96 h. Any deviation from standard culture conditions is indicated below.

### 2.3. Biomass estimation

The culture broth was filtered through preweighed Whatman no. 1 filter paper and the culture filtrate used as the source of extracellular lipase. The filter paper containing the biomass was dried for 48 h at 80°C and its dry weight estimated.

### 2.4. Lipase activity

Lipase activity in the culture filtrates was determined by titrimetry (using olive oil as substrate) [8] and *p*-nitrophenyl palmitate assay [9]. One unit of lipase activity is defined as the amount of enzyme required to release 1 μmol of fatty acid or *p*-nitrophenol at 37°C under standard assay conditions (pH 7.0, reaction time 30 min).

Various parameters were studied in order to achieve maximum lipase production from *Aspergillus terreus*.

1. Selection of oil: 21 different oils, namely, olive, corn, sunflower, vegetable, mustard, linseed, tallow, coconut, groundnut, castor, soybean, karanja, kusum, watermelon, amla, almond, gourd, neem, jasmine, rose and shikakai were evaluated for lipase production at a concentration of 1.5% v/v. Each subsequent factor was examined after taking into account the previously optimised condition.
2. Concentration of corn oil: lipase production was studied at corn oil concentrations varying from 0.5 to 3.0% v/v.
3. Effect of various emulsifiers: various emulsifiers, namely, gum acacia, polyvinyl alcohol (at a concentration of 10% v/v) Tween 80, Tween 40 and Tween 20 (at a concentration of 1% w/v) were used to emulsify the substrate to study their effect on lipase production.
4. Effect of temperature: temperatures ranging from 25 to 50°C were tested for their effect on lipase production by *A. terreus*.
5. Effect of pH: the production medium was adjusted using 0.1 M citrate phosphate for pH values between 3.0 and 6.0, phosphate buffer for pH value 7.0, Tris–HCl for pH 8.0 and 9.0 and glycine–NaOH for pH value 10.0 in order to study the effect of pH on lipase production by *A. terreus*.
6. Effect of various nitrogen sources: the effect of various inorganic and organic nitrogen sources at a concentration of 0.2% w/v was studied on lipase production.
   - Organic nitrogen sources: casein hydrolysate, peptone, beef extract, yeast extract, corn gluten meal, casein, tryptone, asparagine, aspartic acid.
   - Inorganic nitrogen sources: ammonium chloride, ammonium nitrate, ammonium dihydrogen ortho phosphate, ammonium sulphate.
7. Effect of various sugars and sugar alcohols as additives(0.2% w/v):
   - Monosaccharides: fructose, xylose.
   - Disaccharides: lactose, sucrose, maltose.
   - Polysaccharides: dextran, pectin, starch, carboxy methyl cellulose.
   - Sugar alcohol: mannitol, sorbitol.
   - Glycerol.
8. Effect of $Ca^{2+}$ and $Mg^{2+}$ ions: $Ca^{2+}$ and $Mg^{2+}$ ions in the range 0.1–2 mM were studied for their effect on lipase production.

### 2.5. Synthesis of esters by *A. terreus* lipase

Crude culture filtrate containing lipase of *A. terreus* produced under optimised conditions was subjected to ammonium sulphate precipitation (85% saturation). The resulting precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0) and dialysed against the same buffer. The partially purified lipase sample was lyophilised and used for ester synthesis.

Two milli-litres of 200 mM of fatty acids (butyric, caproic, caprylic, capric, myristic, palmitic, stearic and oleic) solution were mixed with 2 ml of 50 mM of sugar/sugar alcohol (glucose, fructose and sorbitol), aliphatic alcohol (methanol, iso-propanol, iso-amyl alcohol) or ascorbic acid mixture in hexane in 15 ml screw capped vials. 50 mg of the lyophilised powder (containing 15 U/mg lipase activity) was added to the reaction mixture. The reactants were incubated at 40°C in a shaker at 200 rpm for 24 h. The products were analysed qualitatively by thin layer chromatography using silica gel plates (Merck) using solvent system petroleum ether:diethyl ether:acetic acid, 80:30:1. The products were quantitated by titrating the remaining fatty acids in the reaction mixture with 0.01 N NaOH and expressing the results in terms of conversion of fatty acids to esters.

## 3. Results and discussion

*A. terreus* produces an extracellular thermostable lipase on olive oil as a carbon source [4]. Since olive oil is an expensive component of any economic lipase production medium, in the present investigation its replacement by other oils was attempted. Among the various oils tried, maximum lipase production (1640 U/l) by *A. terreus* was achieved using corn oil (Table 1) which is much cheaper than olive oil. Corn oil has also

Table 1
Selection of best oil for lipase production by *A. terreus*[a]

| Oil | Biomass (g dry weight/50 ml) | Lipase production (U/l) |
|---|---|---|
| Olive | 0.51 | 1380 |
| Corn | 0.56 | 1640 |
| Sunflower | 0.55 | 910 |
| Vegetable | 0.65 | 300 |
| Mustard | 0.56 | 830 |
| Linseed | 0.48 | 530 |
| Tallow | 0.55 | 1090 |
| Coconut | 0.48 | 1090 |
| Groundnut | 0.48 | 950 |
| Castor | 0.31 | 610 |
| Soybean | 0.36 | 330 |
| Karanja | 0.20 | 157 |
| Kusum | 0.19 | 126 |
| Watermelon | 0.26 | 680 |
| Amla | 0.62 | 1150 |
| Almond | 0.17 | 495 |
| Gourd | 0 30 | 971 |
| Neem | 0.30 | 894 |
| Jasmine | 0.40 | 1430 |
| Rose | 0.35 | 842 |
| Shikakai | 0.57 | 875 |

[a] The results are a mean of three replicates repeated twice.

been used for the production of thermostable, alkaline lipase from *Bacillus* strain A30-1 [10]. Sufficient amounts of lipase were produced on jasmine, tallow, coconut and sunflower oils. Lipase production by *A. terreus* was thus possible using both saturated and unsaturated fatty acid containing oils.

Studies on varying concentrations of corn oil in an emulsified state showed that lipase production improved to 1790 U/l using 2% v/v corn oil (Fig. 1) after which it showed a decline. These results are in accordance with studies on *Hendersonula toruloidea* [11] where lipase production decreased at 3.8% v/v olive oil concentration. Micelles of the lipidic substrates are one of the important parameters for lipase production as they increase the surface area of contact of the substrate with the organism. Among various emulsifiers tried, gum acacia proved to be the best emulsifier and further increased lipase production to 2450 U/l (Fig. 2).Gum acacia has also been reported to support good lipase production from *Rhizopus oligosporus* [12].

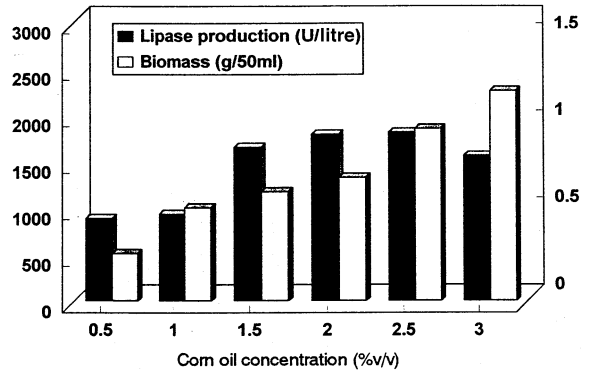


Fig. 1. Lipase production by *A. terreus* at various concentrations of corn oil.

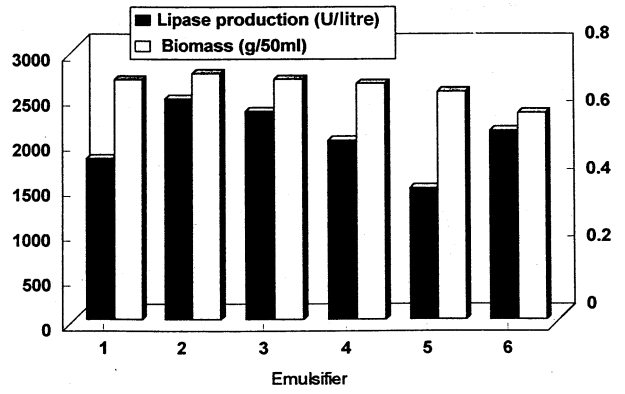


Fig. 2. Effect of emulsifiers on lipase production, 1, control (without emusifier); 2, gum acacia; 3, Tween-80; 4, Tween-40; 5, Tween-20; 6, Triton X-100.

*A. terreus* was capable of producing lipase in the range of 25–45°C with maximum production at 37°C. However, the organism failed to grow at 50°C (Fig. 3). Thermophiles such as *Humicola lanuginosa* [13] however showed high temperature optima (40–45°C) for growth and lipase production.

Maximum growth and lipase production (4010 U/l) was achieved at pH 9.0 by *A. terreus* (Fig. 4) although pH 4.0–10.0 supported both growth and lipase production. At pH 7.0 and 8.0 significant lipase was also produced but dropped significantly at pH 3.0. Thus pH 9.0 was selected for further studies.

Among the various organic and inorganic nitrogenous sources tested as additives, casein at a concentration of 0.1% w/v (Figs. 5 and 6) enhanced both growth and lipase production (6130 U/l). Corn gluten meal was the next best which increased lipase production to 5390 U/l (Fig. 5). Among various inorganic nitrogen sources tested none increased lipase production significantly. Hence the concentration of sodium nitrate was further

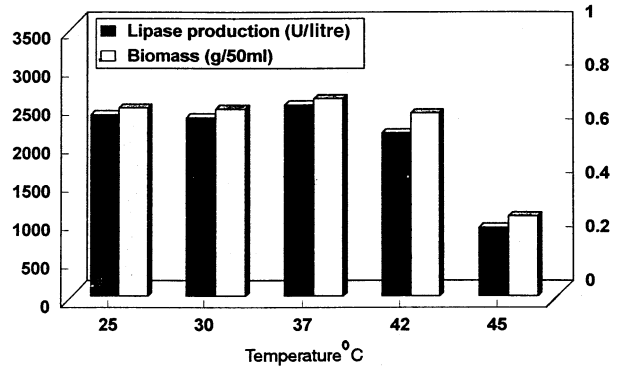


Fig. 3. Effect of temperature on lipase production.

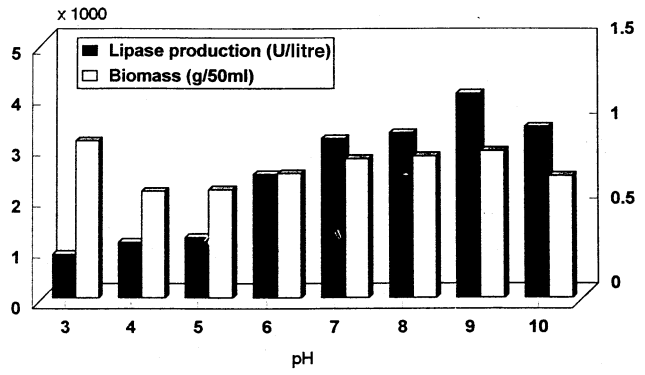


Fig. 4. Lipase production at various pH values.

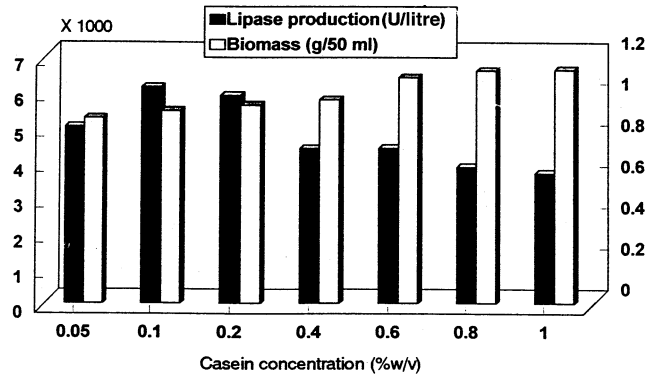


Fig. 6. Effect of various concentrations of casein on lipase production.

standardised. It was possible to decrease the concentration of $NaNO_3$ from 0.6 to 0.2% w/v without affecting enzyme production. Lipase production actually increased to 6350 U/l (Fig. 7). None of the sugars or sugar alcohols further enhanced lipase production. Most of the sugars like maltose, lactose and xylose significantly decreased growth as well as lipase production.

Calcium ions at 1.0 mM concentration caused a further increase in lipase production to 7320 U/l (Table 2). Lipase production and release was also regulated by $Ca^{2+}$ ions in the case of *Fusarium oxysporum* [14]. Magnesium ions at 1.5 mM concentration further increased lipase production to 7780 U/l (Table 2) and thus showed a calcium-dependent secretion mechanism. However, in both the cases there was no effect on the growth of the fungus. Exocytosis of proteins in eukaryotes occurs through 'regulated secretion' pathways controlled by free $Ca^{2+}$ or $Mg^{2+}$ ions concentration [14]. Thus a 5.72-fold increase in lipase production by *A. terreus* was obtained on optimising various nutritional and physical factors.

Lipase from *A. terreus* is capable of carrying out a wide variety of esterification reactions as is evident from Tables 3–5. These esters have important potential applications in detergents, food, pharmaceuticals and oleochemical industries [7,15–17]. Of the various sugars and sugar alcohols tested for esterification with various fatty acids, more than 50% conversion rates were obtained in the case of all fatty acids using sorbitol (Table 3) by *A. terreus* lipase. Maximum yields of 80% were obtained with sorbitol and sucrose esters of stearic acid (Table 3). Similar results have been reported in the case of fructose and sucrose esters of stearic acid with lipase from *Candida* sp. [18] and *Mucor meihei* [19]. Sorbitol stearate has potential usage as a biosurfactant in detergents and emulsifiers in personal care products [7]. Thus esterification of all fatty acids except oleic acid was possible with all sugars and sugar alcohols. More than 60% conversion yields were obtained in the case of both methanol and iso-amyl esters of all fatty acids except oleic acid. In the case of iso-propanol, high conversion yields were obtained with longer chain fatty acids (Table 4). In the case of synthesis of ascorbyl esters of fatty acids, the longer chain fatty acids viz. palmitic, stearic and oleic acids were preferred (Table 5). This property of *A. terreus* lipase can be utilised for the synthesis of ascorbyl esters of higher chain fatty acids

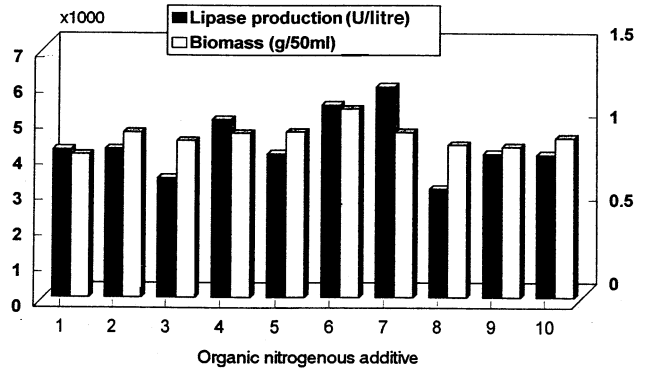


Fig. 5. Effect of organic nitrogenous sources as additives (0.2% w/v) on lipase production, 1, control (without additive); 2, casein hydrolysate; 3, peptone; 4, beef extract; 5, yeast extract; 6, corn gluten meal; 7, casein; 8, tryptone; 9, asparagine; 10, aspartic acid.

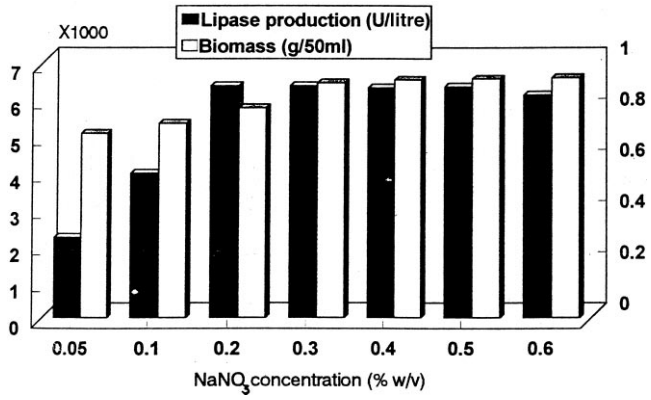


Fig. 7. Effect of various concentrations of sodium nitrate on lipase production.

Table 2
Effect of $Ca^{2+}$ and $Mg^{2+}$ ions on production of *A. terreus* lipase[a]

| Concentration of ion (mM) | Lipase production (U/l) | |
|---|---|---|
| | $Ca^{2+}$ | $Mg^{2+}$ |
| Control 1[b] | 6150 | – |
| Control 2[c] | – | 7320 |
| 0.2 | 6690 | 7320 |
| 0.6 | 6970 | 7590 |
| 1.0 | 7320 | 7730 |
| 1.5 | 7010 | 7780 |
| 2.0 | 6930 | 7090 |

[a] The results are a mean of three replicates repeated twice.
[b] Medium without $Ca^{2+}/Mg^{2+}$ ions.
[c] Medium with $Ca^{2+}$ (1.0 mM) and no $Mg^{2+}$ ions.

which are used as anti-oxidants in food and personal-care products [20]. High conversion yields of 68% have also been reported in the case of synthesis of ascorbyl-palmitate by *Candida antartica* lipase [20]. No esterification with lower chain fatty acids was observed except with butyric acid (conversion yield 56.3%). In contrast to data in Tables 3 and 4 where esters with oleic acid were not formed, reversal in substrate specificities were observed as regards short chain fatty acids excepting butyric acid when ascorbic acid was used as the substrate (Table 5). This variation is well known for lipases which vary markedly in their substrate specificities with a change in one of the substrates and the reaction system [3,15,16].

It can be concluded that lipase production by *A. terreus* is affected by the type of oil, pH and nitrogen source in a growth-dependent manner and by $Ca^{2+}$ and $Mg^{2+}$ in a growth-independent manner. This lipase is able to carry out esterification reactions of industrial importance and is thus a potential industrial biocatalyst.

Table 3
Synthesis of fatty acid esters of sugar alcohol and sugars by*A. terreus* lipase in 24 h at 40°C[a]

| Fatty acid | Sorbitol | % Conversion | |
|---|---|---|---|
| | | Sucrose | Fructose |
| Butyric | 76 | 67 | 68 |
| Caproic | 66 | 71 | 31 |
| Caprylic | 80 | 0 | 76 |
| Capric | 60 | 39 | 74 |
| Myristic | 55 | 46 | 0 |
| Palmitic | 61 | 67 | 70 |
| Stearic | 81 | 80 | 0 |
| Oleic | 0 | 0 | 0 |

[a] The results are a mean of three replicates repeated twice.

Table 4
Synthesis of alcohol esters of fatty acids using *A. terreus* lipase in 24 h at 40°C[a]

| Fatty acid | Methanol | % Conversion | |
|---|---|---|---|
| | | Iso-propanol | Iso-amyl alcohol |
| Butyric | 62 | 33 | 70 |
| Caproic | 41 | 16 | 13 |
| Caprylic | 70 | 14 | 69 |
| Capric | 71 | 32 | 50 |
| Myristic | 79 | 51 | 62 |
| Palmitic | 64 | 50 | 62 |
| Stearic | 66 | 62 | 65 |
| Oleic | 0 | 0 | 0 |

[a] The results are a mean of three replicates repeated twice.

Table 5
Synthesis of ascorbic acid esters of fatty acids by *A. terreus* lipase in 24 h at 40°C[a]

| Fatty acid | % Conversion |
|---|---|
| Butyric | 56 |
| Caproic | 0 |
| Caprylic | 0 |
| Capric | 0 |
| Myristic | 0 |
| Palmitic | 76 |
| Stearic | 69 |
| Oleic | 57 |

[a] The results are a mean of three replicates repeated twice.

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